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Compartmentalization of small ruminant lentivirus between blood and colostrum in infected goats

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Abstract

The compartmentalization of small ruminant lentivirus (SRLV) subtype A (Maedi-Visna virus) and B (caprine arthritis–encephalitis virus) variants was analyzed in colostrum and peripheral blood mononuclear cells of four naturally infected goats. Sequence analysis of DNA and RNA encompassing the V4-V5 *env* regions showed a differential distribution of SRLV variants between the two compartments. Tissue-specific compartmentalization was demonstrated by phylogenetic analysis in three of the four cases. In these animals colostrum proviral sequences were clustered relative to the blood viral sequences. In one goat, the blood and colostrum-derived provirus sequences were intermingled, suggesting trafficking of virus between the two tissues or mirroring a recent infection. Surprisingly, the pattern of free virus variants in the colostrum of all animals corresponded only partially to that of the proviral form, suggesting that free viruses might not derive from infected colostral cells. The compartmentalization of SRLV between peripheral blood and colostrum indicates that lactogenic transmission may involve specific viruses not present in the proviral populations circulating in the blood.
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Keywords: Animal model; Colostrum; Compartmentalization; Lentivirus; Small ruminant lentivirus; SRLV

Introduction

Small ruminant lentivirus (SRLV) include Maedi-Visna virus (SRLV subtype A) and caprine arthritis–encephalitis virus (SRLV subtype B) and cause persistent infections that, in about one third of the animals, progress to induce inflammatory and degenerative pathological processes in their target organs, for instance the mammary gland, the carpal joints, the central nervous system and the lungs. The ingestion of contaminated colostrum and milk immediately after birth is considered the principal source of infection for kids, while horizontal transmission may play an important role in older animals. A small proportion of monocytes is infected and virus replication is triggered by the maturation of monocytes to macrophages. The number of infected monocytes has been reported to steeply increase at the end of gestation, in concomitance with the onset of lactation (Milhau et al., 2005).

Intrauterine infections are considered to be rare events in small ruminants and the ingestion of infected colostrum is the principal source of infection for the newborn kids. Therefore, infection and replication of SRLV in the mammary gland are of pivotal importance to permit the spread of these viruses from one generation to the next. The monocyte/macrophage lineage and dendritic cells are the main target cells for caprine arthritis–encephalitis virus (CAEV) and Maedi-Visna virus (MVV) but several other cell types, such as mammary epithelial cells can also be infected (Lerondelle et al., 1999).

In a recent study, Milhau and colleagues (2005) observed that endothelial cells, mature and immature luminal epithelial cells, fibroblasts and myoepithelial cells from goat mammary gland biopsies were susceptible to CAEV infection *in vitro*, with different levels of sensitivity depending on the type of cell infected. Carrozza et al. (2003) demonstrated by immunohistochemistry the presence of MVV in interstitial fibroblasts, acinar epithelial cells, macrophages, endothelial cells, adipocytes and desquamating epithelium or macrophages in the lumen of the mammary acini. Thus, not only macrophages but also

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mammary epithelial cells may have a role in replication and as a reservoir of the virus in this organ and in MVV pathogenesis (Bolea et al., 2006).

All these cells may act as a reservoir for the virus and play an important role in virus dissemination and in the pathogenesis of lentivirus-induced mastitis. Additionally, these cells may be a source of locally replicating viruses distinct from the quasi-species detected in the peripheral blood.

Recent studies of HIV-1-infected patients have demonstrated an apparent compartmentalization of viral envelope quasi-species among different host tissues (Gupta et al., 2000; Becquart et al., 2002; Zhang et al., 2002). In particular, Becquart et al. have demonstrated a compartmentalization of HIV-1 between breast milk and peripheral blood of infected mothers and, within the breast milk, between free virus and provirus. Additionally, in a macaque-SIV model of virus transmission by breast-feeding, the authors found evidence that the viral genotypes selectively transmitted to infants by breast-feeding represent variants that may be particularly adapted for expression in milk (Rychert et al., 2006). The importance of the lactogenic transmission of SRLV, and the fact that these viruses do not induce an immunodeficiency syndrome that may influence the distribution of particular viruses in different compartments, make SRLV a particularly suitable model for studying this form of vertical virus transmission. To date there is no study of SRLV *env* variation or compartmentalization in naturally infected goats between peripheral blood and milk, where potential evolution of specialized *env* species might influence the transmission of infection from mother to kid via colostrum and milk.

Five major regions of sequence diversity between strains (V1-V5) have been described in the caprine arthritis-encephalitis lentivirus (CAEV) envelope surface unit glycoprotein (SU) (Valas et al., 2000). Studies of CAEV variation during persistent infection in experimentally infected goats have clearly identified diverse and dynamic changes in envelope sequences (Hotzel et al., 2002). The above-mentioned studies defined two discrete regions of CAEV SU that undergo rapid sequence variation in persistently infected goats; this may play an important role in virus-host interactions.

In this study we analyzed, at the phylogenetic and phenetic level, the distribution of envelope nucleotide sequences in blood and colostrum. We present evidence that the viral genotypes present in the colostrum are distinct from those found in the blood and may be specific for this epidemiologically most relevant compartment.

Results

Characteristics of the study population

None of the four goats showed clinical signs attributable to lentiviral infection. PBMCs from each goat were co-cultivated with GSM cells and virus was isolated from all these samples. The number of proviral DNA and viral RNA copies per μg of total genome was determined by minimum χ^2 for limit dilution assays. Controls for PCR included genomic DNA isolated from an SRLV negative goat spiked with 1, 5, 10, or 50 copies of a SRLV A- or SRLV B-plasmid DNA, as well as reagent and SRLV-negative controls. The limit of detection for the first round of PCR was 5 copies of SRLV A- or SRLV B-plasmid, whereas the nested approach had a sensitivity of 1 copy (data not shown).

The proviral and viral copy numbers differed between the four animals and the compartments analyzed (Table 1). Analysis of proviral load in PBMC showed that goats #12, #658 and #666 had a low provirus load (mean 140 copies/μg) whereas goat #13 manifested a higher provirus load (9.8×10^3 copies/μg). Proviral load in CSC was higher in all 4 animals compared to PBMC. Furthermore, viral RNA was detected in the CSC and as a free virus in colostrum whey of every goat. The highest viral expression levels were observed in the CSC of goat #13, with values close to 10^5 copies/μg. Intracellular viral load was similar to the proviral load in the same colostral sample, whereas free virus was at limit of detection (<10 copies/μg) in goats #12, #13 and #658, whereas goat #666 was characterized by a higher cell-free viral load (450 copies/μg).

Sequence analysis

A 610-bp region, corresponding to the V4-V5 regions of SU and the N-terminal stretch of the transmembrane portion of the SRLV *env* gene, was amplified from blood and colostrum (DNA and RNA) and sequenced to determine the viral genetic diversity in 4 subjects and two compartments (Table 2). A total of 360 clones were analyzed: 180 from PBMC samples, 90 from CSC and 90 from colostrum whey.

Table 1
Percentage of monocytes (m) in blood and percentage of epithelial cells (E) and macrophages (M) in infected goats and correspondent PCR results according to the different compartments (blood and colostrum)

Goat	SRLV	Blood copies/μg				Colostrum copies/μg			
		PBMC			Plasma	Somatic cells			Whey
		% m	DNA	RNA	RNA	% cells	DNA	RNA	RNA
12	B/A	3.3	222	–	–	M 62 E 4	2.2×10^3	4.2×10^3	<10
13	B/A	3.3	9.8×10^3	–	–	M 49 E 22	41.9×10^3	99.7×10^3	<10
658	B	4.4	157	–	–	M 63 E 7	9.6×10^3	12.1×10^3	<10
666	B	2.5	40	–	–	M 60 E 0	32.6×10^3	33.7×10^3	417

Number of viral and proviral copies in 1 μg of total genome in the different compartments (blood and colostrum) calculated by minimum χ^2 for limit dilution assays.

Table 2
Nucleotide substitution and genetic variability intra- and inter-compartment

Goat	Compartment	*ds*	dn	ds/*dn*	Intra-compartment variability	Inter-compartment variability [a]	LMA [b]	r^2
					Mean %±SE	Mean %±SE		
12	PBMC DNA	0.04	0.01	4.82	1.3±0.3			
	CSC DNA	0.02	0.01	3.50	1.0±0.2	2.6±0.3	50.8	0.124
	CSC RNA	0.03	0.01	3.94	1.8±0.2	2.2±0.4	55.2	0.208
13	PBMC DNA	0.02	0.01	2.07	1.2±0.3			
	CSC DNA	0.05	0.01	4.00	3.1±0.3	2.3±0.3	86.5	0.285*
	CSC RNA	0.04	0.02	3.16	1.9±0.4	2.2±0.4	81.5	0.172*
658	PBMC DNA	0.14	0.02	6.61	3.0±0.5			
	CSC DNA	0.15	0.02	7.20	1.1±0.2	4.8±0.7	100	0.673*
	CSC RNA	0.08	0.03	4.21	3.7±0.5	4.6±0.6	87.8	0.489*
666	PBMC DNA	0.11	0.06	2.40	3.1±0.4			
	CSC DNA	0.1	0.03	3.32	3.5±0.5	4.1±0.5	70.6	0.191*
	CSC RNA	0.06	0.02	2.96	3.0±0.4	3.2±0.4	67.8	0.161*

Compartmentalization analysis between compartments has been performed by likelihood mapping analysis (LMA) and Mantel's test (r^2=Pearson correlation).
[a] Mean nucleotide divergence between compartments: PBMC DNA×CSC DNA and PBMC DNA×CSC RNA.
[b] Percentage of quartets supporting compartmentalization.
* $P<0.001$.

The percentage of divergence at the nucleotide level among the PBMC-derived clones from goat #12 was 15.7±1.3%. The proviral population detected in the blood of this goat was divided into two main clusters: cluster I, related to SRLV B strains, was characterized by a mean nucleotide variability of 0.4±0.2% and cluster II, closely related to SRLV A strains, showed a mean nucleotide variability of 1.3±0.3%.

The PBMC-derived clones from goat #13 diverged at the nucleotide level by 19.3±1.4%, with sequences belonging to two different populations: cluster I, closely related to SRLV B strains, was characterized by a mean nucleotide variability of 1.2±0.3%, whereas the mean distance within cluster II, comprising a group of sequences related to SRLV A strains, was 2.9±0.5%.

Analysis of colostrum-derived proviral and viral sequences from both goats #12 and #13 revealed the presence of only one SRLV strain despite the presence of co-infection in blood. SRLV subtype A was found in the colostrum of goat #12, whereas in the colostrum of goat #13 only the SRLV subtype B was detected. Moreover, 100 colostrum-derived clones from both DNA and RNA were tested by means of a heteroduplex mobility assay (HMA). PCR products derived from a SRLV B-like and a SRLV A-like clone were used as reference strains. This analysis confirmed the homogeneity of the colostral samples, demonstrated by the presence of heteroduplexes with mobility close to the homodimers formed by only one reference strain (data not shown). Analysis of 100 blood-derived clones with HMA showed a 66% of SRLV subtype A and a 85% of SRLV subtype B sequences in goats #12 and #13, respectively (data not shown). The SRLV subtype present in the colostrum corresponded to the dominant subtype detected in the blood by this analysis.

The average variability of SRLV A proviral and viral sequences in the colostrum of goat #12 was 1±0.2% and 1.8±0.2%, respectively, whereas variability of SRLV B proviral and viral sequences in the colostrum of goat #13 was 2.1±0.3% and 1.9±0.4%, respectively.

The sequence analysis among compartments of goat #12 revealed a mean divergence between blood and colostrum proviral sequences of 2.6±0.3%, whereas mean distances of colostrum viral sequences from blood and colostrum proviral sequences were 3±0.3% and 1.4±0.2%, respectively.

Mean divergence between blood and colostrum proviral sequences of goat #13 was 2.3±0.4%, whereas mean distances of colostrum viral sequences from blood and colostrum proviral ones were identical (2.2±0.4%).

Sequence analysis within compartments of the two other goats (#658 and #666) revealed the presence of only one SRLV subtype B population. The mean percentage of divergence among proviral variants in PBMC of goat #658 was 3±0.5% whereas in colostrum the proviral and viral variability was 1.1±0.2% and 3.7±0.45%, respectively. The mean percentage of divergence among proviral variants in PBMC of goat #666 (3.1±0.4%) was very similar to that in colostrum for both proviral and viral variants (3.5±0.5% and 3.0±0.4%, respectively).

The sequence analysis among compartments of goat #658 showed a mean divergence between blood and colostrum provirus of 4.8±0.7%. RNA viral sequences isolated in colostrum revealed a mean nucleotide distance to proviral sequences in blood and colostrum of 4.6±0.6% and 3.4±0.5%, respectively. In goat #666, mean nucleotide distance between the proviral sequences of the two compartments was 4.1±0.5%, whereas mean percentages of nucleotide difference between colostrum-derived viral sequences and the proviral population in both blood and colostrum were very similar (3.2±0.4% and 3.6±0.5%, respectively).

Nucleotide substitutions

Tissue-specific compartmentalization can result from selection of a genotype by the specific environment of that compartment, or by a "founder" effect due to the isolated replication of a few genotypes. To determine if the viral sequences detected in one or both compartments were evolving under a positive

selective pressure, we monitored the relative rates of nucleotide substitution at synonymous and nonsynonymous sites (*ds* and *dn*). Indeed, a greater *dn* compared to *ds* would indicate the presence of positive selection and support the hypothesis of tissue-specific adaptation and selection (Sharp, 1997). We determined average *dn* and *ds* for proviral and viral sequences obtained from blood and colostrum. The *dn* rates were lower than the *ds* rates for all compartments. In all animals, the sequences from both compartments yielded *ds*/*dn* ratios of a similar magnitude, suggesting a relatively homogeneous selective pressure in all of the compartments analyzed. A signature pattern analysis was performed in search of conserved amino acid motifs linking colostrum- and blood-derived sequences. The first approach calculated (with the VESPA software) the frequency of an amino acid at a specific position and then determined whether there was a distinct pattern for one set of sequences (i.e., blood or colostrum). No convincing signature pattern was associated exclusively with colostrum or blood sequences.

The second approach used Shannon entropy (with the ENTROPY software) to calculate the consistency of an amino acid at one specific position. To assess the statistical significance of the most distinctive motifs identified, a Monte Carlo-like randomization of viral sequences was used (Korber et al., 1994). The greatest entropy values clustered in a five-amino acid-long discrete region (Fig. 1) previously described (Hotzel et al., 2002) and designated hypervariable region 2 (HV2). Fig. 1 shows the aligned amino acid sequences of proviral and viral clones derived from both compartments around this HV2 region.

We also compared the number of potential N-linked glycosylation sites between colostrum and blood sequences by the Wilcoxon rank-sum test (*N*-glycosite tool, Zhang et al., 2004). The V4 region of SRLV A and B strains from the goats in this study contains five conserved N-linked glycosylation sites (495NWT, 505NCS, 511NAT, 529NCS, 536NES, numbered according to the amino acid sequence of the CAEV-CO strain, Saltarelli et al., 1990) in all sequences from both colostrum and blood. We found no significant difference between the two sets of sequences, except for goat #658, where colostrum-derived proviral and viral sequences were characterized by one additional, conserved N-linked glycosylation site (519NGT), not present in the blood proviral set.

Phylogenetic analysis

As described (see Materials and methods), we employed a combination of limiting dilution and end-point PCRs, followed by cloning and sequencing in order to exclude the re-sampling of proviral sequences that is known to occur during these PCR-cloning-sequencing procedures (Liu et al., 1996).

Sequences derived from the PBMC of two animals (#12 and #13) grouped in two distinct clusters related to SRLV subtypes A and B. These co-infection events are described in detail in a separate paper (Pisoni et al., 2007). In contrast, only one subtype, A or B, was found in the colostrum of goats #12 and #13, respectively. A recombinant virus containing both subtype A and B sequences in its *env* sequence was also detected in the blood and colostrum of goat #12 (Pisoni et al., 2007). The sequences from the two other animals (#658 and #666) grouped with SRLV subtype B reference sequences in both blood and colostrum (data not shown). *Env* sequences derived from a given animal consistently clustered together, excluding any cross-contamination between samples derived from different animals (supported by 100% of the bootstrap samples). Figs. 2A, B, C and D depict the phylogenetic trees generated with sequences belonging to the same SRLV subtype and obtained from blood and colostrum for each of the four goats.

In three of the four data sets, the colostrum-derived proviral sequences were tightly clustered relative to the blood-derived proviral sequences (the strongest case for tissue-specific compartmentalization of virus can be made for goat #658). In the other data set (goat #12), the blood- and colostrum-derived proviral sequences were intermingled.

In goats #13 and #666, a minor blood proviral variant clustered (bootstrap values greater than 80%) with colostrum proviral and viral sequences.

The viral sequences from colostrum formed subclusters within the radiation of proviral sequences from the same compartment. This colostrum-specific grouping was evident for goats #13 and #666 (Figs. 2B and D). For the other goats (Figs. 2A and C), a similar tissue-specific grouping was also observed, particularly in goat #658, where a sub-cluster of colostrum-derived viral sequences appears to emerge from the group of blood proviral sequences.

Phylogenetic compartmentalization

The compartmentalization of the SRLV quasi-species was evaluated in the PBMC and CSC (Table 2). In particular, likelihood mapping of separate groups of PBMC and colostrum proviral sequences revealed the significant grouping of PBMC quasi-species in goats #13, #658 and #666 (the percentage of quartets supporting this topology varied from 70 to 100%). Likelihood mapping analysis (LMA) for compartmentalization of colostrum viral sequences revealed a significant clustering in the same goats but with a lower percentage of quartets supporting this topology (from 68 to 88%). In goat #13 no compartmentalization of colostrum viral sequences was observed.

To obtain statistical confirmation of genetic compartmentalization, we used Mantel's test to search for a relation between pairwise Tamura-Nei distances and compartment distribution. Significant genetic compartmentalization was observed for blood variants in goats #13, #658 and #666, since 1000 random permutations of the *Mc* matrix did not produce a correlation coefficient higher than that found with the observed distribution ($P<0.001$). The coefficients of the Pearson correlation for these goats were $r^2=0.29$, $r^2=0.67$ and $r^2=0.19$, respectively. The high P value of Mantel's test demonstrated formally that sequences from PBMC were closer to each other than to any sequences from the colostrum. This was not the case for quasi-species in the blood of goat #12 ($r^2=0.1$, $P=0.05$), compared with colostrum proviral quasi-species.

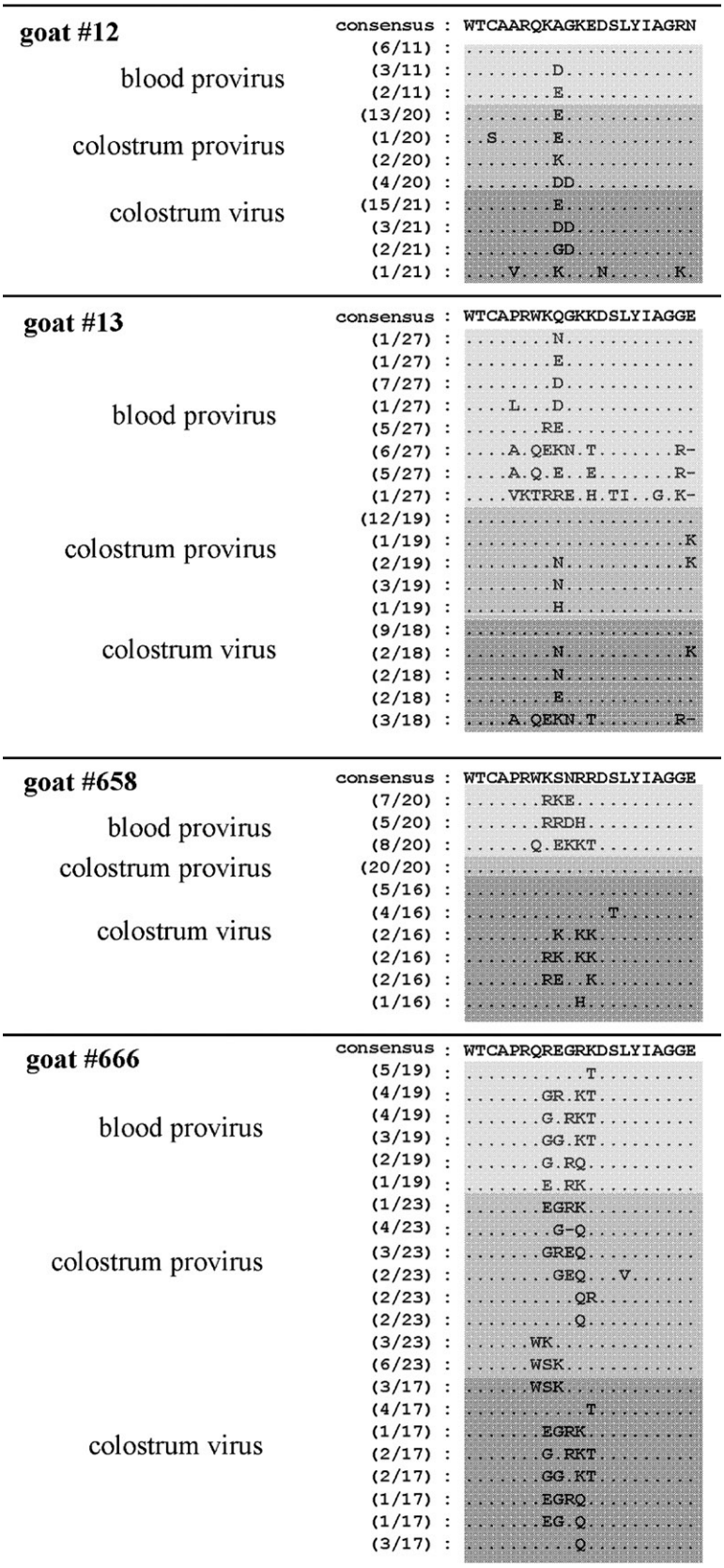
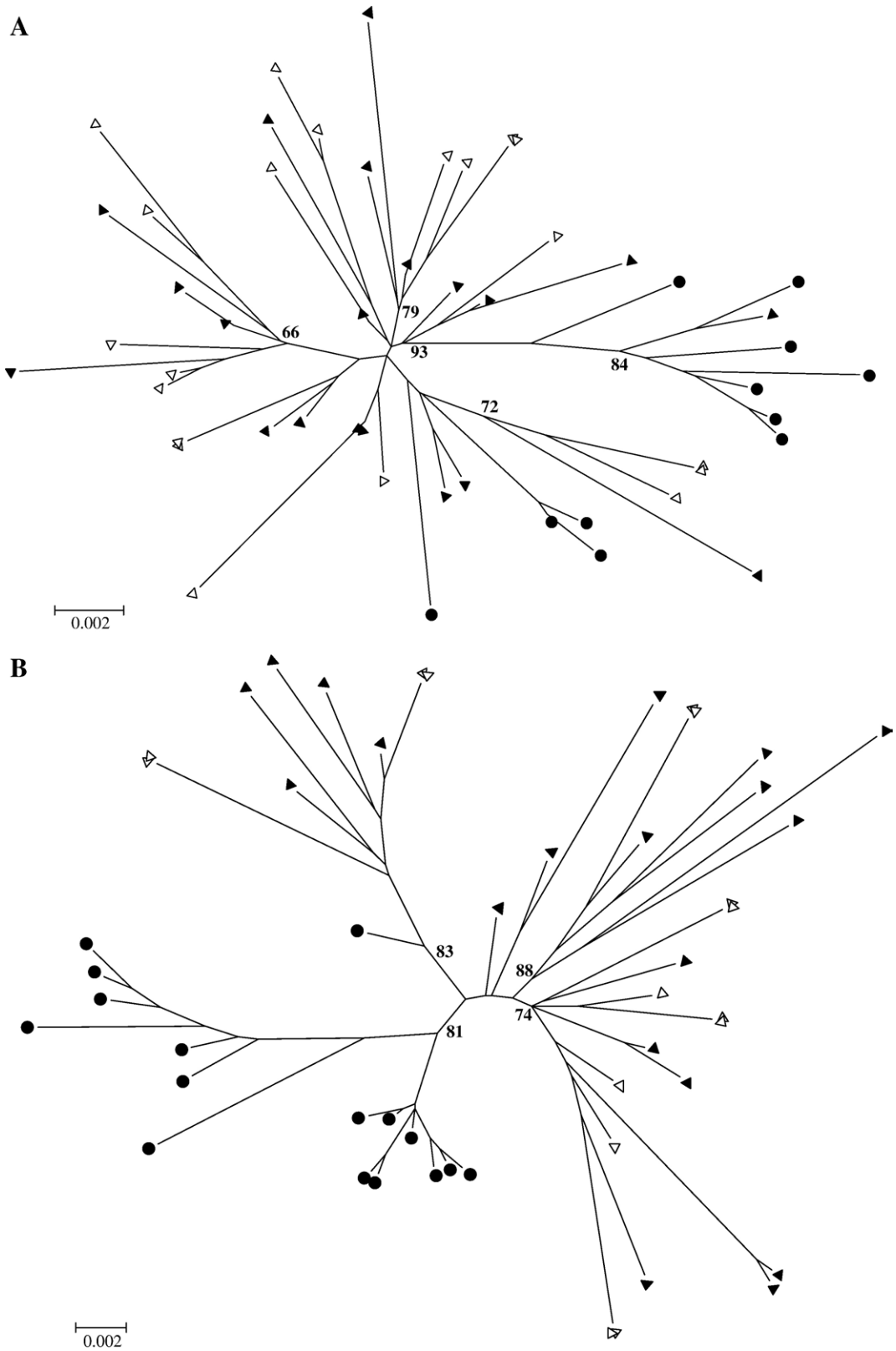
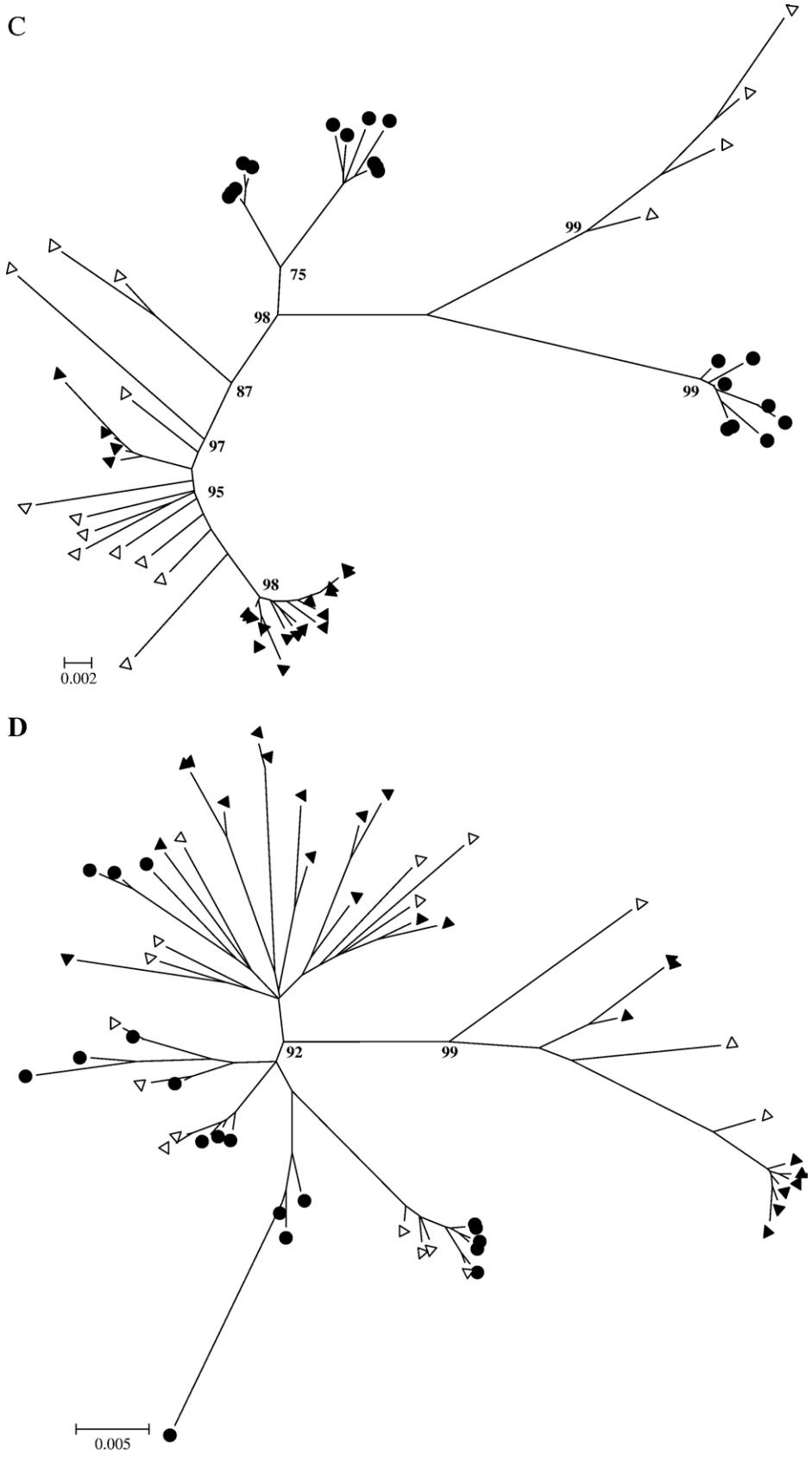


Fig. 1. Deduced amino acid sequences of SRLV hypervariable region 2 (HV2) in SU protein from the four goats. The amino acid sequences were aligned with the consensus sequences obtained from both blood and colostrum from each animal. Different gray shadings indicate sequences obtained from blood provirus, colostrum provirus and virus. The numbers in parentheses represent the number of clones with identical sequence/total number of clones sequenced from that compartment. Dots indicate identity with consensus sequences and dashes indicate deletions.



Figs. 2. (A–D) Phylogenetic trees of envelope clones derived from the blood and colostrum of all goats, constructed with the neighbor-joining method. Trees from A to D are representative for goats #12, #13, #658 and #666, respectively. For goats with co-infection (#12 and #13), only sequences of virus isolated in both blood and colostrum are represented in phylogenetic trees A (MVV-like sequences) and B (CAEV-like sequences). Bootstrap values of key branch nodes are indicated (1000 data sets). A full circle represents blood-derived proviral sequences, a full triangle represents colostrum-derived proviral sequences, an open triangle represents colostrum-derived viral sequences.



Figs. 2 (*continued*).

Discussion

Following infection, the retrotranscribed SRLVs genome integrates as a provirus in the chromosomes of the monocytes and remains latent until these cells mature to macrophages (Narayan et al., 1983; Gendelman et al., 1986). Infected macrophages are abundantly present in colostrum and milk, which are considered the main sources of natural transmission (Radostits et al., 2000; Preziuso et al., 2004; Peterhans et al., 2004). The induction of mastitis by SRLV may favor virus transmission to the suckling kids by increasing the number of infected cells in colostrum and milk (Clements and Zink, 1996). The strategic choice of SRLV to target the mammary gland permits an efficient transmission of these viruses to the next generation and their persistence at the population level. The importance of the lactogenic transmission for SRLV makes these viruses a perfect model to study this type of infection in lentiviruses. Additionally, the investigation of the viral genotypes present in various compartments may reveal the presence in the mammary gland of distinct viruses particularly adapted to the lactogenic transmission. In this study we observed large differences in proviral load at parturition in the blood compartment between the different goats. Goat #13 had a blood proviral load that was 100 times higher than that of the other three animals. These differences were also observed by Ravazzolo et al. (2006), who showed that goats experimentally infected with a molecularly cloned virus (CAEV-CO) exhibit extremely different viral loads possibly due to the influence of the genetic background on the efficiency of the immune response. We found a remarkable difference of proviral load between the blood and the colostrum. For all goats, colostrum cells were collected and analyzed by 24 h after parturition and in this compartment we found by far the highest proviral and viral load, with up to 10^5 copies of viral RNA in colostral cells. This is in perfect agreement with the observation by Ravazzolo et al. (2006) in experimentally infected animals that the mammary gland is a privileged site of virus replication *in vivo* and with the concept that colostrum is the main source of infection for kids (Peterhans et al., 2004; Blacklaws et al., 2004).

In contrast, the goats had only between 1 and 10 copies of viral RNA in cell-free colostrum. As described for HIV, virus inactivating substances known to occur in the colostrum may lower the free virus load and explain the discrepancy between high cell associated viral load and low free viral load in this compartment (Van de Perre, 2000; Kazmi et al., 2006). It is now demonstrated that different SRLV subtypes can infect both sheep and goats without restriction (Shah et al., 2004; Pisoni et al., 2005) and that co-infection may happen under natural conditions (Pisoni et al., 2007). Information about the tissue distribution of MVV and CAEV in co-infected goats, however, is missing. Interestingly, the two goats in the present study (#12 and #13) were characterized by a different distribution of MVV and CAEV between blood and colostrum. The detection, within the sensitivity limit of our assays, of only one viral and proviral species in the colostrum, strongly suggests that the barrier between the blood and the mammary gland may represent a bottleneck exerting a strong selection on the viruses colonizing this compartment. It is noteworthy that, in both cases, the viral genotype found in the colostrum corresponded to the dominating genotype detected in the blood, suggesting that the aforementioned bottleneck may not select particular virus genotypes but just represent a stochastic barrier favoring the passage of the dominant genotypes present in blood.

In this study, we demonstrate a compartmentalization of SRLV between colostrum and blood in infected goats. Extensive genetic characterization of RNA and DNA variants amplified from colostrum and blood revealed distinct patterns of distribution of viral populations.

Like other lentiviruses, SRLV productively infects cells of the monocyte-macrophage lineage, which distribute the viral infection throughout the body. The provirus-bearing monocytes carry the virus in the blood and, following extravasation, differentiate into various tissue macrophages, where virus replication takes place (Gendelman et al., 1986; Ravazzolo et al., 2006). As an upstream precursor, the blood monocyte compartment may actually seed virus-infected macrophages in these organs and thereby establish the reservoir. This may also be the case for the mammary gland and colostrum, where virus-infected monocytes entering from the blood may be the principal source of virus-infected macrophages. Alternatively, colostral macrophages may also enter the glandular epithelium of the mammary gland from inductive sites of the mucosal-associated lymphoid tissue and mammary lymph nodes (Outteridge and Lee, 1988).

The compartmentalization of sequences observed between blood and colostrum in goats #13, #658 and #666 favors the hypothesis that the colostral cells were infected with a particular set of SRLV variants. The actual source of these viral sequences is unknown; however, the presence in goat #658 of a sub-cluster of colostrum sequences phylogenetically related to blood proviral sequences, strongly proposes the blood as the original source of these viruses. This is also supported by the observations that in goats #13 and #666 a minor blood proviral variant clustered with colostrum proviral and viral sequences. The origin of the colostral sequences phylogenetically unrelated to blood-derived sequences is unknown and these viruses may have originated from local mucosal sites. As discussed above, goat #12 showed a strict compartmentalization between blood and colostrum with regard to the subtype of virus present in the mammary gland, where only subtype A (MVV) was detected. Within this subtype, however, no compartmentalization was observed between blood and colostrum, neither for proviral nor for viral sequences. Lack of compartmentalization and a relative homogeneity of sequences in different tissues are characteristics for the acute phase of infection in HIV (Zhang et al., 2002) and may explain the sequence distribution in this goat. This goat, however, is 5 years old and, considering that in SRLV an infection at birth is most probable, the most likely explanation is that just by chance the viruses that colonized the mammary gland are still circulating in the blood in the form of provirus.

The potential factors influencing compartmentalization and differential tissue distribution of SRLV genotypes remain to be defined. As for other lentiviruses, mutation-prone replication favors the genetic diversity of SRLV that is high albeit lower

than in HIV. Our finding that SRLV sequences differed between blood and colostrum implies that some degree of independent replication and/or evolution occurred, as proposed for HIV (Zhang et al., 2002). Selective migration of viral variants particularly suited to replicate in the mammary gland is unlikely, as we did not find a common mammary gland "signature pattern" such as that described for brain-derived sequences in HIV (Korber et al., 1994). The fact that we sequenced only a short region of *env*, however, does not exclude that such a "signature pattern" may be present in a different region of Env.

Furthermore, there were no significant differences in the numbers or positions of N-linked glycosylation sites between colostrum and blood sequences except in one goat. The SU4 variable region contains five conserved N-linked glycosylation sites and four near cysteine residues in the same position as in the CAEV-CO reference strain, which confirms the observation by Valas et al. (2000). It has been proposed that the SU4 variable region of CAEV and MVV forms a highly constrained and surface-exposed domain and that a cysteine loop may have an analogous function to the V3 principal neutralizing domain of HIV-1 (Knowles et al., 1991; Skraban et al., 1999). Most of amino acid substitution and the greatest values of entropy were found within this region, particularly in the so-called hypervariable region 2 (HV2). This may well be related to the immune pressure exerted on this region by neutralizing antibody, as proposed by Skraban et al. (1999).

We support the hypothesis of a parallel evolution of viral variants, following the infiltration of the mammary gland with a "founder virus", possibly representing the major variant circulating in the blood at the time of colonization of this organ, as observed for the two co-infected animals, #12 and #13.

The initial mechanisms driving compartmentalization may be quite disparate and reflect a selective pressure for a particular cell tropism, e.g., for mammary epithelial cells, or be related to the actual immune pressure exerted on the viruses in this compartment.

Milhau et al. (2005) have shown that different cell types isolated from mammary tissue, including luminal epithelial cells, myoepithelial cells, endothelial cells and fibroblasts, are highly susceptible to *in vitro* infection. Additionally, they demonstrated that, *in vitro*, the infection of mammary myoepithelial cells is restricted to the integration of proviral DNA without viral expression. The authors propose that the latently infected myoepithelial cells are a potential virus reservoir *in vivo* that may be reactivated during mammogenesis, contributing to the selection of particular virus variants. Moreover, the interactions between infected monocytes, different cells of the mammary gland and immunocompetent cells influence the regulation of viral expression during natural infections, thereby controlling the passage of infected cells into colostrum and milk (Le Jan et al., 2005).

In conclusion, in this work we present the first analysis of SRLV sequences conducted in parallel in blood- and colostrum-cells. We demonstrated the presence of different viral *env* sequences in the sites analyzed. Elucidating the relationship between genetic evolution of SRLV in specific anatomical sites and the biological properties of the selected Env sequences will lead to a better understanding of the significance of these adaptive changes in the context of SRLV pathogenesis, immune control and, particularly, virus transmission. The animals described in this work are now pregnant and in a dry period. We will re-analyze the virus quasi-species distribution during the next lactation period, especially in the colostrum, and characterize the viruses transmitted to the kids.

Materials and methods

Animals

This study involved four seropositive and naturally infected Saanen goats from two different dairy farms (farms A and B) with a seroprevalence of 90–100% for SRLV infection. Samples were taken from the animals 1 month before delivery for serological and molecular diagnosis of SRLV infection. ELISA test was performed with Pourquier kit, molecular characterization was done with PCR amplification as previously described (Pisoni et al., 2005). Animals from farm A were naturally infected by SRLV subtype B1 (data not shown) whereas animals from farm B were characterized by co-infection with SRLV subtypes A and B (Pisoni et al., 2007).

After delivery, blood and serum samples were collected from each goat. At the same time colostrum samples (milk secretion during the first 24 h after delivery) were collected for somatic cells and whey isolation.

Blood and colostrum samples

Ten milliliters of blood from each animal was collected in vacutainers without anticoagulant for serum isolation. Fifty milliliters of blood from each animal was collected in 2 mM EDTA vacutainers. A differential leucocyte count was done using a hemocytometer (Melet Schloesing MS4). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples and purified by centrifugation through Ficoll-Paque Plus (GE Healthcare Europe GmbH, Milan Italy), as previously described (Pisoni et al., 2007). One hundred milliliters of colostrum was collected from goats after delivery. Colostrum somatic cells (CSC) were obtained after dilution 2 to 4 in sterile PBS, centrifugation at 4 °C, 800×*g* for 15 min. The lipid layer was discarded, and the clear supernatant was aspirated into a separated tube. The cells collected as pellets were washed twice with PBS. Differential cell counts were estimated by means of an esterase stain (Yam et al., 1971).

Viral isolation

Goat synovial membrane (GSM) cells, originally derived from the explanted carpal synovial membrane of a colostrum-deprived newborn goat (Narayan et al., 1989), were resuspended into 10 ml of tissue culture medium (MEM-Earle; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and seeded into 25 cm^2 tissue culture flasks at a density of 5×10^5 cells/ml and incubated in a CO_2 incubator at 37 °C for 4 to 6 h. Approximately 5×10^6

PBMCs were resuspended in 10 ml of tissue culture medium (MEM-Earle; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), seeded on adherent GSM and incubated overnight at 37 °C in a CO_2 incubator. Non-adherent cells were removed by washing twice with MEM-Earle without additives, and the cultures were further incubated for 10 days. The medium was changed at intervals of 3 to 4 days. Cells were passaged at weekly intervals for a maximum of six passages. Cytopathic effects indicative of virus replication such as the presence of syncytia or cell lysis were monitored upon fixing the cells with a solution of 10% formalin and staining with May–Grünwald–Giemsa solutions. When maximum CPE was observed, cells and medium were harvested and stored at −80 °C for DNA and RNA extraction.

DNA and RNA extraction and quantification

DNA was extracted from PBMC and CSC pellets containing $\leq 5\times10^6$ cells using commercial silica-gel spin-columns selective for genomic DNA (QIAamp DNA Blood Mini Kit, QIAGEN), according to the manufacturer's instructions. RNA was extracted from PBMC and CSC pellets containing $\leq 10^7$ cells, from blood plasma and colostrum whey using TRIZOL reagent (Invitrogen, Milan, Italy). RNAs were immediately retro-transcribed using SuperScript III RNase H-free RT (Invitrogen, Milan, Italy). DNA and cDNA, diluted in fivefold series and in triplicate, were used for semi-nested PCR to amplify SRLV *env* sequences (see below). After limiting-dilution PCRs, SRLV DNA and cDNA copies were quantified with the computer program QUALITY (Rodrigo et al., 1997), a variant of the minimum χ^2 method for limiting dilution assays. The copy number is estimated by the value that maximizes the goodness of fit between the observed numbers of negative reactions and the expected numbers of negative reactions (the latter estimated using a Poisson probability distribution) as measured by the χ^2 statistic.

PCRs and cloning of PCR fragments

Cellular DNAs from PBMC and CSC, and cDNAs, which were reverse transcribed from PBMC, CSC, plasma, whey and fat viral RNAs, were used in semi-nested PCRs to amplify a 607 bp fragment encompassing the C-terminal part of surface (SU4-SU5 or V4-V5) and N-terminal part of transmembrane (TM) proteins of *env* gene (7482–8089 bp of CAEV-CO, *M33677*) with the following primers: #563, #564 and #567. The primers and PCR conditions used were described previously (Mordasini et al., 2006). To avoid template re-sampling (Liu et al., 1996), we performed limiting-dilution PCRs (see above).

PCR amplicons were separated on 2% agarose gels, and bands of the expected sizes were excised. The excised fragments were purified with Perfectprep Gel Cleanup kit (Eppendorf, Milano, Italy) and subsequently cloned with the TOPO TA cloning kit (pCR 4-TOPO Vector; Invitrogen, Italy) according to the manufacturer's protocol. Fifty to 100 colonies were picked and grown overnight at 37 °C in LB medium (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl) with 50 μg/ml ampicillin (SERVA GmbH, Heidelberg, Germany). Minipreps were prepared with the Wizard Plus Kit (Promega, Madison, WI) according to the manufacturer's instructions.

Sequencing and sequences analysis

Sequencing reactions were performed by CRIBI Services (CRIBI, Padova, Italy) on an ABI377 sequencer by using the ABI PRISM dye-terminator cycle sequencing ready reaction kit with Amplitaq DNA polymerase (Perkin-Elmer, Applied Biosystems). The sequences obtained were edited and analyzed with the following software: BioEdit (biblio), ClustalW (Thompson et al., 1994) and Genedoc (version 2.5). Pairwise genetic distances were calculated by using MEGA version 2.1 (Kumar et al., 2001) with the Tamura-Nei substitution model, applying the default setting, with the exception that all sites with ambiguous codes and gaps were ignored. Synonymous/nonsynonymous ratios (*ds*/*dn*) were calculated using the SNAP program (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html) (Korber, 2000). A signature pattern analysis was done to search for possible amino acid positions that would provide a conserved pattern within the colostrum *env* sequences relative to the blood *env* sequences. The first approach calculated (with VESPA software, Korber and Myers, 1992) the frequency of an amino acid at a specific position and then determined whether there was a distinct pattern for one set of sequences (i.e., blood or colostrum). The second approach used Shannon entropy $H(i)$ (with ENTROPY software, Korber et al., 1994) to calculate the consistency of an amino acid at one specific position. To assess the statistical significance of the most distinctive motifs identified, a Monte Carlo-like randomization of viral sequences was used to test the statistical significance of the signature pattern (Korber et al., 1994).

Phylogenetic analysis

For each individual, phylogenetic trees were constructed using the neighbor-joining (NJ) method implemented in MEGA with the Tamura–Nei gamma distance (Tamura and Nei, 1993). The statistical confidence of the topologies was assessed with 1000 bootstrap replicates (Felsenstein, 1985). The shape parameter alpha for a discrete gamma distribution of substitution rates and the transition/transversion rate ratio parameter kappa were estimated simultaneously by maximum likelihood using Yang's BASEML program implemented in the PAML (Phylogenetic Analysis by Maximum Likelihood) program package (Yang, 1996).

Phylogenetic compartmentalization

The phylogenetic approach was aimed at evaluating the evolutionary relationship between SRLV quasi-species on the basis of the compartmental origin of the clones. The significance of the topology obtained was evaluated by means of bootstrap (see above) and maximum-likelihood analyses.

Likelihood mapping analysis (LMA) is a quartet puzzling method based on the maximum-likelihood approach designed to investigate *a priori* the phylogenetic information contained in a sequence alignment (without computing an overall tree). The LMA program is implemented in TREE-PUZZLE (Schmidt et al., 2002). The method is based on calculating the likelihood of all of the possible fully resolved tree topologies for each analyzed quartet (groups of four randomly chosen sequences). Each quartet has three possible topologies: the likelihood of each is estimated by using the maximum-likelihood approach, and the likelihood values are represented as a dot inside an equilateral triangle. Each corner of the triangle represents one of the three fully resolved trees; the center of the triangle being a completely unresolved tree topology (star-like evolution) and the sides being two equally possible topologies. The percentage of dots in each area is a measure of the probability of the topologies: dots falling in a corner support the corresponding topology, whereas a high percentage of dots in the center indicates an unresolved topology (all of the topologies are equally possible). When analyzing a set of sequences, it is possible to group them in different subsets and the method indicates the most likely topology among them. The likelihood mapping analysis was made by grouping the sequences on the basis of their compartment of origin (a=blood, b=colostrum).

Phenetic compartmental analysis

Whereas molecular phylogenetic studies determine ancestral relationships among sequences, phenetic analyses determine the degree of genetic similarity among sequences. To determine if sequences from any compartment shared more genetic identity with each other than with sequences from other compartments (or time points), we used Mantel's test (Smouse et al., 1986; Waddle, 1994), a generalized regression permutation procedure, which compares two distance matrices. One distance matrix consists of pairwise Tamura–Nei gamma distance of sequences from all compartments obtained at a given time point. The second matrix, M_c, is an idealized matrix of the same dimensions such that $M_c(i,j)=0$ if sequence i is from the same compartment as sequence j, 1 otherwise.

The statistic test is the square of the Pearson correlation coefficient, r^2, computed over all pairs of elements, excluding the diagonal, of both matrices. If sequences from each compartment are more similar to other sequences in that compartment than to sequences from different compartments, then r^2 will be high. The null distribution was constructed by permuting the rows and columns of the idealized matrix 1000 times and counting the number of times the value of r^2 is exceeded. The hypothesis that there is compartmental phenetic structure is rejected if more than 5% of the permutations exceed r^2.

Nucleotide sequence accession number

All new sequences were deposited in the GenBank database and are available under accession number EF685709 to EF685902.

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